



Year: 2018

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Abstract: BACKGROUND: Angiostrongylosis is considered as emerging disease in dogs in Belgium. Detection of first-stage larvae in feces using the Baermann method has an imperfect sensitivity. **OBJECTIVES:** Investigation of efficacy of noninvasive blood and fecal diagnostic tests in comparison with PCR on bronchoalveolar lavage (BAL) material in a small series of coughing or dyspnoeic dogs naturally infected with *Angiostrongylus vasorum*. **ANIMALS:** Seven dogs with angiostrongylosis. **METHODS:** Retrospective study. Dogs with cough, exercise intolerance and dyspnea of 2- to 8-week duration. Diagnostic methods used included Baermann analysis, AngioDetect rapid assay, ELISAs for detection of circulating antigen and specific antibodies and qPCR on BAL material. **RESULTS:** Baermann analysis, AngioDetect rapid assay, antigen ELISA, antibody ELISA, and qPCR on BAL material were positive in 3/7, 2/7, 3/6, 6/6, and 7/7 dogs, respectively. ELISA for antibodies or qPCR on BAL material were essential for definitive diagnosis in 3 dogs. Relative sensitivities of AngioDetect rapid assay, Baermann analysis, and ELISA for antigen detection were lower than 50% compared with ELISA for antibodies or qPCR on BAL material. **CONCLUSION AND CLINICAL IMPORTANCE:** In this small clinical series, Baermann analysis and AngioDetect rapid assay failed to confirm the diagnosis in some dogs. Therefore, ELISA for antibody detection and qPCR on BAL material should strongly be considered in clinically suspected dogs when antigen detection methods (AngioDetect or ELISA) and Baermann analysis are negative.

DOI: <https://doi.org/10.1111/jvim.15092>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-158972>

Journal Article

Published Version




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Originally published at:

Canonne, A Morgane; Billen, F; Losson, B; Peters, I; Schnyder, Manuela; Clercx, C (2018). Angiostrongylosis in dogs with negative fecal and in-clinic rapid serological tests: 7 cases (2013-2017). *Journal of Veterinary Internal Medicine*, 32(3):951-955.

DOI: <https://doi.org/10.1111/jvim.15092>

Angiostrongylosis in dogs with negative fecal and in-clinic rapid serological tests: 7 Cases (2013-2017)

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Objectives: Investigation of efficacy of noninvasive blood and fecal diagnostic tests in comparison with PCR on bronchoalveolar lavage (BAL) material in a small series of coughing or dyspnoeic dogs naturally infected with *Angiostrongylus vasorum*.

Animals: Seven dogs with angiostrongylosis.

Methods: Retrospective study. Dogs with cough, exercise intolerance and dyspnea of 2- to 8-week duration. Diagnostic methods used included Baermann analysis, AngioDetect rapid assay, ELISAs for detection of circulating antigen and specific antibodies and qPCR on BAL material.

Results: Baermann analysis, AngioDetect rapid assay, antigen ELISA, antibody ELISA, and qPCR on BAL material were positive in 3/7, 2/7, 3/6, 6/6, and 7/7 dogs, respectively. ELISA for antibodies or qPCR on BAL material were essential for definitive diagnosis in 3 dogs. Relative sensitivities of AngioDetect rapid assay, Baermann analysis, and ELISA for antigen detection were lower than 50% compared with ELISA for antibodies or qPCR on BAL material.

Conclusion and Clinical Importance: In this small clinical series, Baermann analysis and AngioDetect rapid assay failed to confirm the diagnosis in some dogs. Therefore, ELISA for antibody detection and qPCR on BAL material should strongly be considered in clinically suspected dogs when antigen detection methods (AngioDetect or ELISA) and Baermann analysis are negative.

KEYWORDS

AngioDetect, bronchoalveolar lavage, qPCR, serology

1 | INTRODUCTION

Angiostrongylus vasorum (*A. vasorum*) is a metastrongyloid nematode residing in the right cardiac ventricle and the pulmonary arteries of dogs and foxes. The latter wild carnivores are considered as the main reservoir and many gastropod species such as slugs and snails act as

intermediate hosts.¹ Over the last 5 years, several studies confirmed the presence of *A. vasorum* in most countries of western Europe. In Belgium, angiostrongylosis in dogs is now considered as an emerging disease.²⁻⁴ Clinical signs are various, nonspecific and potentially fatal if left untreated; thus, an early diagnosis is essential.⁵⁻⁸

An old and still the most widely used method for the diagnosis is based on the detection of first-stage larvae by the Baermann fecal technique and has a suboptimal sensitivity.⁹⁻¹² A direct fecal smear can be also used in general practice; but, despite its attractive rapidity, its

Abbreviations: BAL, bronchoalveolar lavage; qPCR, quantitative polymerase chain reaction.

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sensitivity is also poor.¹³ Higher sensitivity is obtained by the FLOTAC technique but it is unfrequently used despite the additional advantage that it can be applied also with older samples.¹⁴

Other diagnostic methods including serological or molecular assays have been successively developed to improve diagnosis. Detection of specific antibodies and detection of specific circulating *A. vasorum* antigen by ELISA are sensitive and highly specific.^{15–18} They have been validated for reliable diagnosis^{19,20} and both, antibody and antigen ELISA, are offered by the Institute of Parasitology, Vetsuisse Faculty, University of Zurich, Switzerland. An in-clinic rapid ELISA (AngioDetect rapid assay, *Idexx Laboratories*) for antigen detection with a high specificity has then been developed. However, direct comparison with the ELISA detecting circulating antigens showed a delay of ~3–4 weeks for antigen detection by the AngioDetect rapid assay.²⁰ Furthermore, only anecdotic information about sensitivity of the AngioDetect rapid assay in naturally infected dogs with negative Baermann test is reported.^{4,21}

Quantitative PCR test (qPCR) has been developed and was successively used on different substrates including blood, feces, and tracheal swabs from dogs with experimental infection^{22,23} and from naturally infected foxes.²⁴ Circulating DNA in the blood was evaluated as an early indicator of infection, though has a low sensitivity,^{23,24} while detection of specific circulating parasitic antigens by ELISA was positive in all dogs from 7 weeks after infection, which corresponds to the period before patency.²³

Recently, qPCR on bronchoalveolar lavage (BAL) material was described to identify dogs infected with *A. vasorum* in a series of coughing dogs³; however, its relative diagnostic value in comparison with fecal and serological tests has not been investigated. The aim of this study was to report and compare results obtained by the Baermann fecal technique, by serological detection of circulating *A. vasorum* antigen adopting a rapid assay (AngioDetect rapid assay) and ELISA, by serology for detection of specific antibodies against *A. vasorum* by ELISA and by qPCR on BAL material in a small series of dogs diagnosed with angiostrongylosis.

2 | MATERIALS AND METHODS

Client-owned dogs with signs of respiratory disease, including cough, exercise intolerance or respiratory distress, presented at the University Veterinary Small Animal Teaching Hospital of Liège between March 2013 and April 2017, diagnosed with angiostrongylosis and for which results of different diagnostic methods were available, were retrospectively recruited. The five diagnostic methods included the Baermann technique, the rapid immunochromatography assay on blood or plasma (AngioDetect rapid assay, *Idexx Laboratories*), ELISA for detection of circulating antigens of *A. vasorum* and specific antibodies and qPCR performed on BAL material.

Angiostrongylosis was suspected based on compatible clinical signs, radiologic findings, bronchoscopy and cytological examination of BAL material; diagnosis was confirmed by a positive result of qPCR on BAL material and clinical response to anthelmintic treatment including

fenbendazole (50 mg/kg q24h PO for 3 weeks) or moxidectin (2.5 mg/kg repeated after 2–4 weeks).

The Baermann technique was performed at the Laboratory of Parasitology of the Faculty of Veterinary Medicine, University of Liège, on three consecutive samplings from each dog as previously described; differentiation between first-stage larvae of *A. vasorum* and *Crenosoma vulpis* was based on morphological criteria (characteristic notch feature on the tail for *A. vasorum*).^{9–12}

From each dog, one or two serum samples were frozen at the diagnosis. One aliquot of serum from each dog was retrospectively thawed and a rapid test detecting circulating *A. vasorum* antigen (AngioDetect rapid assay, *Idexx Laboratories*, Westbrook, Maine) was performed, according to the manufacturer's instructions. Another aliquot of serum from 6 dogs was sent to the Institute of Parasitology, Vetsuisse Faculty, University of Zurich, Switzerland, and further analyzed for detection of specific circulating *A. vasorum* antigen and specific antibodies against *A. vasorum* by previously validated ELISAs. Circulating adult *A. vasorum* antigens were detected by a sandwich-ELISA using monoclonal and polyclonal antibodies, as previously described.¹⁸ Specific antibodies were detected by a sandwich-ELISA using *A. vasorum* adult somatic antigen purified by monoclonal antibodies (mAb Av 5/5) as previously detailed.¹⁶ For both ELISAs, absorbance values were read at 405 nm with a Multiscan RC ELISA reader (Thermo Labsystems, Helsinki, Finland). All test runs included a background control, a conjugate control, three positive control sera from three experimentally infected dogs and two negative control sera from uninfected dogs. Cut-off values (OD = 0.287 and OD = 0.234 for ELISA assay detecting circulating adult antigen and specific antibodies, respectively) were based on the mean value of optical density plus three standard deviations of 2000 randomly selected dog samples (Schnyder, unpublished data).

Bronchoscopy, BAL and laboratory processing of BAL material were performed as previously described.³ All dogs were anesthetized, using various anesthetic protocols including premedication, a 5-minutes preoxygenation period, IV induction and IV maintenance; oxygen saturation was controlled during procedure and recovery with pulse oximetry monitoring. qPCR analysis was performed on uncentrifugated BAL fluid. Genomic DNA (gDNA) was purified from 200 µL of lavage fluid using the DNeasy Blood and Tissue Kit (QIAGEN, Manchester, UK), with the DNA eluted in 100 µL, and qPCR analysis performed on 5 µL gDNA as described previously.^{3,22} This assay has previously been shown to have 100% specificity for *A. vasorum* when DNA from *C. vulpis*, *Eucoleus aerophilus*, *Toxocara canis*, *Dirofilaria immitis*, *Dirofilaria repens*, and *Angiostrongylus cantonensis* were used as controls.

3 | RESULTS

Seven dogs of various breed, age and sex were included (9 months to 10 years, mean age = 5 years, 5 females, and 2 males). Owners reported cough, exercise intolerance and respiratory distress of variable severity from 2-week to 2-months duration.

Baermann analysis, in-clinic rapid test (AngioDetect rapid assay), ELISA for antigen, ELISA for antibodies and qPCR on BAL material for

gDNA detection were positive in 3/7, 2/7, 3/6, 6/6, and 7/7 dogs, respectively. In the three dogs with negative in-clinic rapid test and for which ELISA for antigen detection was available (dogs numbers 4, 5, and 6), ELISA for antigen was also negative. Dog number 3 delivered an invalid result by the in-clinic rapid test, that is, the control line did not repeatedly develop (rapid test repeated twice) but ELISA for antigen was positive for this dog. Samples positive for gDNA detection in BAL material were also positive for detection of specific antibodies. If ELISA for specific antibodies had not been performed, qPCR on BAL material would have been essential for definitive diagnosis in dog numbers 5, 6, and 7, which presented cough and exercise intolerance from 1- to 4-week duration.

All dogs completely recovered with prescribed anthelmintic treatment; in dog numbers 3 and 4 that had positive Baermann analysis, examination of feces collected after the end of the treatment was negative for L1 larvae.

4 | DISCUSSION

This study reports the investigation of the respective diagnostic usefulness of four non-invasive tests (ie, on blood or feces) compared to an invasive procedure (ie, BAL) in dogs with natural infection by *A. vasorum*. Based on this small series of dogs with naturally occurring angiostrongylosis, detection of specific antibodies by ELISA and of gDNA on BAL material had the best relative sensitivities. Indeed, both techniques were essential to confirm diagnosis in 3 cases with clinical signs of short duration, that is, from 1- to 4-week duration.

Results from the present selection of dogs confirm the lower sensitivity of the in-clinic AngioDetect rapid assay in dogs with positive Baermann analysis, as previously observed in a validation study.²⁰ In dogs with positive Baermann analysis, sensitivity of AngioDetect rapid assay was effectively reported to be of 85%, concomitantly with excellent specificity (100%) regarding different cardio-respiratory helminth infections.²⁰ In a further study, still in relation to Baermann analysis, AngioDetect rapid assay had a sensitivity of 97.1% and a specificity of 89%.²¹ Nevertheless, it must be highlighted that positive AngioDetect rapid assay results were reported in some Belgian dogs with negative Baermann analysis and without any clinical signs compatible with angiostrongylosis⁴; it could suggest possible asymptomatic infestation in some dogs (low worm burden?) or, more simply, false-positive results. Moreover, this in-clinic test can remain positive in up to 7 weeks after start of anthelmintic treatment,²⁰ which might reflect either incomplete clearance of infection or slow elimination of antigens from dead adult worms. These last observations can make the interpretation of the in-clinic test challenging.

Direct comparison between the two assays detecting circulating antigen revealed a delay of 3–4 weeks for antigen detection by AngioDetect rapid assay compared to the ELISA.²⁰ In experimental conditions, ELISA for antigens and AngioDetect were respectively positive in all dogs at 11 weeks and 14 weeks after inoculation whereas fecal Baermann analysis was positive in all cases from 8 weeks.²⁰ In the present population, all dogs with negative AngioDetect rapid assay were

also seronegative for antigen-ELISA detection and negative for Baermann analysis with one exception and all dogs presented signs for 1–8 weeks. Therefore, based on results from the present small series, pursuing with antigen-ELISA assay in suspected dogs with negative AngioDetect rapid assay would have been unhelpful. Interestingly, both antigen-ELISA and AngioDetect rapid assay were negative in dog number 2, whereas the Baermann analysis was positive. This result might be explained by formation of antigen–antibody complexes, which inhibit detection of circulating antigens.^{20,25} To test this hypothesis, serum could have been treated by heat as described but this was not performed in this dog. Moreover, it would have been interesting to test sera from dogs numbers 4–5–6 after heat treatment.

ELISA for detection of specific antibodies has better sensitivity than either serologic tests detecting circulating antigen. Sensitivity and specificity of ELISA detecting specific antibodies are 86% and 99%, respectively.¹⁶ In this cases, relative sensitivity of this test was excellent if positive qPCR on BAL material is to be considered as the gold standard for diagnosis. In experimentally infected dogs, specific antibodies were detected from week 3 in some dogs and from week 5 in all dogs and was the most promising test for identifying dogs at an early stage of infection.²³ Seropositivity for antibodies in dogs with negative antigenemia (AngioDetect rapid assay or ELISA) has thus been observed previously.^{20,23} Detection of specific antibodies by ELISA is, to our knowledge, only offered by the Diagnostic Unit of the Institute of Parasitology, University of Zurich. Moreover, neither Baermann technique nor serological evaluation for antigens and antibodies are suitable tools to evaluate immediate biological response to anthelmintic treatment, because all these tests could, based on data from experimental infections, remain positive for some time: Baermann analysis can persist positive for up to 3 weeks after treatment, while the other tests can be still positive up to 9 weeks, suggesting possible failure to clear the infestation.^{16,23} Lastly, the presence of specific antibodies was observed in Belgian dogs without history or physical examination suggestive of angiostrongylosis⁴ and could be interpreted, once more, as asymptomatic infestation, previous infestation or false positive results. It highlights that any diagnostic test should always be interpreted in light of the epidemiological and clinical context.

This clinical series confirms the clinical usefulness of qPCR on BAL material.⁴ The main disadvantage for this procedure is that it requires the collection of material under anesthesia, which can be particular risky in dogs with respiratory distress. The financial cost of bronchoscopy could also limit its use as a first choice diagnostic procedure. Cytological diagnosis of *A. vasorum* with fine-needle aspiration of the lung under only brief sedation has been reported in occasional case reports in dogs^{26,27} and in one cat with *Aelurostrongylus abstrusus* infection.²⁸ However, the sensitivity of this test has not been investigated in clinical series, especially in comparison with qPCR on BAL material; in addition, absence of peripheral pulmonary lesions on radiographs and potential coagulopathies²⁹ can preclude the realization of this procedure. Bronchoalveolar lavage analysis has the advantage to provide additional diagnostic information such as the presence of bacterial coinfection that requires specific therapeutic management or monitoring.³ Moreover, parasitic coinfection with *C. vulpis* can also be detected

by fecal analysis and BAL material analysis. *C. vulpis* infection was also recently documented as a regularly occurring parasite in coughing dogs in Belgium and coinfection with *A. vasorum* occurs.^{3,4,30,31} The sensitivity of Baermann analysis for diagnosis of *C. vulpis* infections is to our knowledge not described in the literature but intermittent excretion in analogy with *A. vasorum* can be assumed. Differentiating *C. vulpis* from *A. vasorum* infection is relevant because of the higher pathogenicity of *A. vasorum* especially related to associated bleeding diathesis.^{7,8} In this context, treatment of *A. vasorum* infected dogs with tranexamic acid and plasma transfusion could be helpful to normalize hyperfibrinolysis and hypofibrinogenemia occurring in severe cases.²⁹

Limitations of the study include the small-sized clinical series and the lack of qPCR performed on blood or feces. Molecular methods on blood or feces are noninvasive and commercially available. Moreover, blood qPCR provided the earliest indicator of infection, that is, 2 weeks post infection in a previous experimental study, however with low sensitivity.²³ Positive blood qPCR with negative antigenemia was previously reported and possible detection of DNA originating from L3 during migration and maturation is hypothesized to explain this observation.

In this series, positive qPCR on BAL material was used for definitive diagnosis. Only one study has investigated qPCR on BAL material from healthy dogs or dogs with other pulmonary conditions and suggested that false positive results are unlikely.^{3,31} Furthermore, as each dog from our cohort favorably responded to medical management, including fenbendazole or moxidectin, authors were confident in definitive diagnosis of angiostrongylosis in each case and thus, qPCR on BAL appears as a highly specific test.

Finally, as the first inclusion criteria was the presence of signs of respiratory disease (cough, exercise intolerance, or respiratory distress), it is less clear if the qPCR would perform as favorably on BAL from dogs with isolated signs of neurological disease or extrapulmonary hemorrhages secondary to *A. vasorum* infestation. While the life cycle of *A. vasorum* always involve the lungs, some affected dogs fail to present any historical or radiological signs of respiratory lesions; in such situations, performance of qPCR on BAL has not been assessed, even if the procedure is rarely contraindicated. Accordingly, true sensitivity of qPCR on BAL cannot be assessed based on this small cohort of dogs presented for signs of respiratory disease, the lack of any defined gold standard and because positive qPCR was the first inclusion criteria. However, these results of four noninvasive tests compared with qPCR on BAL render this analysis as potential gold standard in dogs with angiostrongylosis and signs of respiratory disease.

5 | CONCLUSION

In conclusion, this study compares four noninvasive diagnostic procedures with the BAL material analysis in naturally infected coughing dogs. While ELISA for detection of specific antibodies and qPCR on BAL material gave highly valuable results, the best procedure for diagnosing angiostrongylosis in dogs that combines noninvasiveness, promptness, sensitivity, and specificity has to be chosen depending on

the individual situation. Although Baermann analysis and the in-clinic rapid test should be used as first-line tools in clinically suspect dogs because of their availability, cost-effectiveness, and inherent noninvasiveness, they might be of lower sensitivity than detection of specific antibodies and qPCR on BAL material, especially in cases of early infection. Based on the comparative usefulness of qPCR on BAL material and ELISA for detection of specific antibodies in this case series, effort should be accomplished for increased execution of tests detecting specific antibodies or by potentially empowering antigen detection methods through heat treatments of sera in dogs suspected of *A. vasorum* infection but negative by first-line tools, prioritizing noninvasive methods.

ACKNOWLEDGMENTS

The authors gratefully thank Dr Maud Girod, Dr Emilie VanGrinsven, Dr Elodie Darnis, Dr Elodie Roels, Dr Valérie VanDerVorst, Dr Charlotte Auquier, Dr Emilie Krafft, and veterinary technicians for their help in the inclusion of cases and the collection of samples. Location of the work was Veterinary Teaching Clinic of Small Animals, Faculty of Veterinary Medicine, University of Liège, Belgium. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. This study was presented as oral communication in the 27th ECVIM Congress, Malta, September 2017.

CONFLICT OF INTEREST DECLARATION

The authors declare that they have no conflict of interest with the contents of this article.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

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How to cite this article: Canonne AM, Billen F, Losson B, Peters I, Schnyder M, Clercx C. Angiostrongylosis in dogs with negative fecal and in-clinic rapid serological tests: 7 Cases (2013–2017). *J Vet Intern Med.* 2018;32:951–955. <https://doi.org/10.1111/jvim.15092>